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The Phosphoenolpyruvate: Sugar Phosphotransferase system is involved in sensitivity to the glucosylated bacteriocin sublancin

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30 **Abstract**

31

32 The mode of action of a group of glycosylated antimicrobial peptides known as glycocins
33 remains to be elucidated. In the current study on one glycocin, sublancin, we identified the
34 phosphoenolpyruvate:sugar phosphotransferase system (PTS) of *Bacillus* species as a key
35 player in bacterial sensitivity. Sublancin kills several Gram-positive bacteria such as
36 *Bacillus* species and *Staphylococcus aureus*, including methicillin-resistant *S. aureus*
37 (MRSA). Unlike other classes of bacteriocins for which the PTS is involved in their
38 mechanism of action, we show that the addition of PTS-requiring sugars leads to increased
39 resistance rather than an increased sensitivity, suggesting that sublancin has a distinct
40 mechanism of action. Collectively, our present mutagenesis and genomic studies
41 demonstrate that, in particular, the histidine-containing phosphocarrier protein (HPr) and
42 domain A of enzyme II (PtsG) are critical determinants for the bacterial sensitivity to
43 sublancin.

44 Introduction

45 Bacteriocins are ribosomally synthesised peptides produced by a wide range of bacterial
46 species. These bacteriocins endow the producing bacteria with a competitive advantage in
47 their respective niche. Many bacteriocins are heavily post-translationally processed during
48 their biosynthesis and these modifications are required for activity (1). Nisin is the best
49 studied bacteriocin and belongs to the lantibiotic family (2). The mode of action of nisin
50 involves binding to lipid II, which prevents further cell wall synthesis, followed by
51 formation of pores within the membrane, leading to membrane permeabilization. Leakage
52 of essential metabolites from these cells results in death of the bacteria. Targeting of lipid II
53 by bacteriocins is a common mechanism of action (3-5). Other mechanisms include the
54 targeting of phosphotransferase systems (6, 7), acting as Trojan horses (8, 9), parasitizing
55 iron-uptake pathways (10), and causing the collapse of membrane potential together with
56 leakage of ions and/or a decrease in intracellular ATP concentrations (11). There is much
57 interest in bacteriocins for use in control of bacterial infections and therefore in their
58 mechanisms of action.

59
60 Sublancin is a bacteriocin produced by the Gram-positive soil bacterium *Bacillus subtilis*
61 168. It is capable of killing several species of Gram-positive bacteria, such as *Staphylococcus*
62 *aureus*, including methicillin resistant *S. aureus* (MRSA) (12). Sublancin is encoded on the
63 SP β prophage as a prepeptide by the *sunA* gene (13). The genes necessary for the synthesis
64 of sublancin are also included in this region and are expressed from two promoters. The
65 biosynthetic operon is made up of five individual genes, which are responsible for
66 producing active sublancin. The *sunT* gene is responsible for the export of sublancin and
67 cleavage of its leader sequence. Two thiol-disulfide oxidoreductases, encoded by *bdbA* (the
68 only gene of the operon that is dispensable for active sublancin production) and *bdbB*, are
69 responsible for creating the two disulphide bonds of sublancin. These disulphide bonds
70 involve four of the five cysteine residues that are present in the sublancin peptide (14). The
71 fifth cysteine residue undergoes glucosylation by the glucosyltransferase encoded by the
72 *sunS* gene (15). The second promoter drives expression of a gene encoding the immunity
73 protein SunI that is also required for the production of active sublancin by protecting the
74 producing organism from sublancin (16).

75

76 Sublancin is one of five bacteriocins that have so far been described as being S-
77 glycosylated. Glycocin F is produced by *Lactobacillus plantarum* (17), ASM1 is produced by
78 *Lactobacillus plantarum* A-1 and is an orthologue of glycocin F with five different residues
79 in the C-terminal tail (18, 19), and the putative products of *Bacillus thuringiensis*,
80 thurandacin A and B, have been produced *in vitro* (20). Sublancin is modified on Cys22,
81 with a β -S-linked glucose (15, 21). For glycocin F an S-linked N-acetylglucosamine (GlcNAc)
82 is added to the C-terminal cysteine residue and the other modification is on an internal
83 serine residue with an O-linked GlcNAc (17, 22). Like glycocin F, thurandacin B is
84 glucosylated on two residues; a cysteine residue undergoes S-linked glucosylation, whereas
85 a Ser is modified with an O-linked glucose moiety (20). Judged by homology searches to the
86 bacteriocin sequence or the glucosyltransferase, many other Gram-positive bacteria appear
87 to potentially encode such bacteriocins with much variation in sequence amongst them (15,
88 17).

89
90 The mechanisms by which glucosylated bacteriocins kill sensitive cells are currently
91 unknown. Previous work has identified several genes in *B. subtilis* and *S. aureus* that alter
92 the sensitivity to sublancin. The *mscL* gene encodes the large mechanosensitive channel
93 and its deletion confers sublancin resistance in both *S. aureus* and *B. subtilis* (23). Addition
94 of increased amounts of NaCl also results in increased resistance to sublancin, presumably
95 due to the MscL channel being forced closed. This observation has led to speculation as to
96 whether sublancin is able to enter the cell through this channel. Interestingly, since the
97 connection between sublancin and MscL was reported, streptomycin has also been
98 reported to use the MscL channel to enter the cell (24). In *B. subtilis* the alternative sigma
99 factor σ^W is known to play a role in the resistance to sublancin through its regulation of the
100 *yqeZ-yqfA-yqfB* operon (25). The role these genes play in resistance to sublancin is
101 unknown, but it is likely to be at the cell surface due to their membrane localisation (26).

102
103 In this study, we demonstrate that the phosphoenolpyruvate:sugar phosphotransferase
104 system (PTS) of *B. subtilis* plays a major role in sensitivity to sublancin. In the case of other
105 bacteriocins where the PTS was found to be involved, addition of the PTS-requiring sugars
106 resulted in increased sensitivity to the respective bacteriocin. However, for sublancin, the
107 addition of PTS-requiring sugars leads to increased resistance, suggesting that sublancin
108 has a distinct mechanism of action.

109

110 **Materials and Methods**

111

112 **Bacterial growth**

113 For all strains used in this study, see Table 1. *B. subtilis* 168, *B. subtilis* ATCC 6633 and *B.*
114 *halodurans* C-125 were grown in Lysogeny Broth (LB) at 37 °C with vigorous shaking (250
115 rpm) and on LB agar plates. *B. subtilis* was also grown on M9 agar plates (M9 as described
116 (27), but with the addition of 1.5 % final concentration agar) with and without the addition
117 of sugars at final concentrations of 0.3 % glucose, 0.4 % malate or 0.4 % citrate as specified
118 below. The LB agar used for sublancin inhibition plate assays did not include NaCl.
119 Antibiotics were used for selection when necessary at the following concentrations:
120 spectinomycin 100 µg/mL, kanamycin 20 µg/mL, phleomycin 4 µg/mL, chloramphenicol 5
121 µg/mL and erythromycin 2 µg/mL. Stock sublancin solutions were prepared using PBS.

122

123 **Production and isolation of sublancin 168**

124 Purification of sublancin from its natural producer, *B. subtilis* 168, was performed as
125 previously reported (15).

126

127 **Strain construction**

128 Chromosomal DNA was prepared from *B. subtilis* 168 using a standard procedure as
129 previously described (28). Deletion mutants in *B. subtilis* 168 were created as described by
130 Tanaka et al. (29) and oligonucleotides used are shown in Table S1 in the supplemental
131 material. *B. subtilis* 168 was transformed using PCR products or chromosomal DNA
132 following a standard procedure as previously described (30).

133

134 **Minimum inhibitory concentration (MIC) determination**

135 MICs were determined by the broth dilution method (31). Serial dilutions of sublancin
136 were prepared in sterile deionized water (SDW). Forty-eight well microtiter plates
137 (Corning Costar) were utilized for both *B. subtilis* ATCC 6633 and *Bacillus halodurans* C-
138 125. The total volume of culture in each well was 300 µL; the experimental wells contained
139 30 µL of 10x stock sublancin at defined concentrations and 270 µL of a 1-in-10 dilution
140 (approximately 1×10^8 colony-forming units (CFU) mL⁻¹) of a culture of indicator strain
141 diluted in fresh LB growth medium. In addition, each plate contained several blanks (270

142 μL fresh growth medium and 30 μL SDW) and control wells (270 μL of untreated 1-in-10
143 diluted culture and 30 μL SDW). The optical density at 600 nm ($\text{O.D.}_{600\text{nm}}$) was recorded at
144 hourly intervals from 0 to 6 h with an additional measurement at 18 h using a BioTek
145 Synergy 4H plate reader. Plates were incubated under vigorous agitation at 37 °C. The
146 readings of triplicate experiments were averaged. Growth curves were developed using
147 control (culture and SDW only) readings to ensure sufficient O.D. changes for accurate
148 inhibition assessment. Curve fits for MIC determination were produced by fitting the data
149 with Origin8.5 software using a dose-response curve with the equation: $y = A_1 + (A_2 - A_1)$
150 $/ (1 + 10^{(\text{Log}x0 - x)p})$, with p = variable Hill slope.

151

152 **Sublancin killing kinetics against sensitive *Bacillus* species**

153 Sensitive cultures were grown to mid log phase in LB medium as described above,
154 transferred to 48-well microtiter plates (Corning Costar) and exposed to sublancin at 1x
155 and 4xMIC. Immediately after the addition of sublancin, the $\text{O.D.}_{600\text{nm}}$ was determined using
156 a BioTek Synergy H4 plate reader. The cultures were incubated for 6 h and the $\text{O.D.}_{600\text{nm}}$
157 was recorded every 30 min. To verify that cells were killed, CFU counting was performed
158 by serial dilution and plating.

159

160

161 **Sublancin sensitivity screen of a gene deletion collection of *B. subtilis***

162 Sublancin-induced growth inhibition assays were performed using the procedure
163 described by Dorenbos et al (14), but with modification to enable the screening of large
164 numbers of strains. Overnight cultures of *B. subtilis* mutants and background control strain
165 were grown in 96-well microtiter plates in a plate shaker at 37 °C with shaking at 800 rpm.
166 Bioassay dishes were prepared with LB agar without adding salt. The plates were
167 thoroughly dried before being divided into 48 squares for inoculation. Cotton swabs were
168 dipped into individual wells of the overnight culture before being spread on the
169 appropriate square. Plates were allowed to dry, before spotting 2 µL of an overnight
170 culture of the *B. subtilis* 168 wild-type strain in the centre of each inoculated square. Plates
171 were incubated overnight and visual analysis was used to determine zones of inhibition
172 that were smaller or larger than that of the background strain. Strains with altered zones of
173 inhibition were checked a further three times to ensure the phenotype was reproducible.

174
175 **Sublancin sensitivity assay in liquid medium**

176 Overnight cultures of *B. subtilis* grown in LB were diluted 1:100 in the same medium and
177 grown to O.D._{600nm} 0.5. The bacteria were then diluted 1:20 in a 96-well microtiter plate
178 before growth was monitored in a Synergy4 Biotek plate reader every 10 min (37 °C, with
179 shaking). When the bacteria reached O.D._{600nm} 0.185 (equivalent to 0.5 for a 1 cm path
180 length), sublancin was added at the desired concentration, before resuming the monitoring
181 of growth. Sugars were added at the following final concentrations: glucose 0.3%, malate
182 0.4%, sucrose 0.3 %, fructose 0.3%, glycerol 0.3%, citrate 0.4%, galactose 0.4% and
183 succinate 0.4%.

184
185 **Membrane integrity assay**

186 *B. subtilis* was grown to O.D._{600nm} 0.5, before purified sublancin was added at different
187 concentrations (100 – 500 nM). As a positive control, nisin was added at 10 nM final
188 concentration and a negative control sample contained no bacteriocin. Samples were taken
189 at 30 and 90 min and prepared for LIVE/DEAD® *BacLight*™ analysis (Molecular Probes,
190 Life Technologies) (32). Samples were monitored by flow cytometry using an Accuri C6.
191 The percentages of cells with intact or reduced membrane integrity were calculated.

192
193 **Propidium iodide uptake**

194 Membrane integrity was also evaluated by measuring the uptake of propidium iodide (PI).
195 *B. subtilis* ATCC 6633 and *B. halodurans* C-125 cultures were grown to a density of 4×10^6
196 cells mL⁻¹ and then diluted with fresh LB medium to an O.D._{600nm} of 0.1. Cells were
197 transferred to tubes containing PI (final concentration 25 µM; Molecular Probes Inc.,
198 Leiden, NL), HEPES (1 mM), and sublancin (0, 0.2, 2.0, 20 µM) or nisin (0, 0.2, 2.0, 20 µM),
199 incubated for 30 min at RT, and analysed. Data acquisition was performed with a BD
200 Biosciences LSR II flow cytometer, using excitation at 488 nm with an argon laser and
201 measurement of emission through a band-pass filter at 695/40 nm. A minimum of 50,000
202 events was detected for each sample, and experiments were performed in triplicate. Data
203 analysis to calculate the geometric mean fluorescence intensity (MFI) of gated cell
204 populations was performed using FCS Express 3.00.0311 V Lite Stand-alone software.

205

206 **Generation of stable sublancin resistant mutants and resistance frequency** 207 **determination**

208 Genetically stable, sublancin resistant mutants were generated by growing the sublancin
209 susceptible strains *B. halodurans* C-125 and *B. subtilis* ATCC 6633 in LB as described above
210 (no additional sugars added) until an O.D._{600nm} of 1.0 (mid log phase, 1 cm light path). The
211 cultures were plated on agar plates containing 1x or 4x their respective sublancin MICs.
212 Distinct colonies were observed by 24 h. Resistant colonies were picked, grown in LB and
213 plated on LB plates containing sublancin at 4xMIC to confirm resistance. This procedure
214 generated genetically stable sublancin mutants. The number of resistant mutants that
215 emerged from each sublancin susceptible culture was obtained by generating a serial
216 dilution of each culture. Each dilution was plated on sublancin-containing plates. The total
217 number of cells was determined by plating an appropriate (10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9})
218 dilution of the cultures on non-selective LB agar medium. Colonies from sublancin-
219 containing and non-selective plates were counted after 24 h of incubation. The resistance
220 frequency was determined as the mean number of resistant cells divided by the total
221 number of viable cells per culture.

222

223 **Single Nucleotide Polymorphism (SNP) detected by whole genome sequencing**

224 Genomic DNA (gDNA) of the wild-type *B. halodurans* C-125 and four different sublancin
225 resistant isolates was extracted using an UltraClean® Microbial DNA isolation kit (MO
226 BIO). The gDNAs thus obtained were sequenced using a HiSeq2000 Illumina sequencer,

227 which generated close to 180 million single-reads per lane, for an overall coverage of 360x
228 for the 5 MB genomes. All libraries were individually barcoded and constructed with the
229 TruSeq Sample Prep kits (Illumina). The SNPs and the corresponding genes for resistant *B.*
230 *halodurans* C-125 were identified. In addition, the wild-type *B. halodurans* C-125 strain was
231 mapped to the published *B. halodurans* C-125 sequence (accession no. NC_002570.2) with
232 CLC Genomics Workbench (CLC bio), using default parameters. A consensus sequence of
233 the wild-type and reference genome was obtained and used for SNP detection in sublancin
234 resistant mutants of *B. halodurans*.

235

236 **PCR amplification and validation of SNPs in sublancin resistant *B. halodurans*** 237 **mutants**

238 PCR validation can serve as an iterative and informative process to modify and optimize
239 the SNP filtering criteria to improve SNP calling (33). Primers flanking SNP-containing
240 genes were synthesized and used for PCR amplification of the respective genes. The
241 mutations reported herein were all confirmed by PCR (Table S2 in the supplemental
242 material).

243

244 ***B. halodurans* C-125 gene expression profile**

245 A culture of *B. halodurans* C-125 was grown in LB at 37 °C with vigorous shaking until mid
246 log phase, at which point the culture was split into two 150 mL cultures with one subjected
247 to a sub-inhibitory concentration of sublancin (0.5xMIC). RNA isolation was performed
248 using the RNeasy mini kit (Qiagen) and subsequently treated with RNase-Free DNase
249 (Qiagen). The RNA was dissolved in RNase-free water and quantified using a NanoDrop
250 2000c spectrophotometer (Thermo Scientific). For each sample (i.e. with and without
251 sublancin), 20 µg of total RNA was isolated from three biological replicates. cDNA synthesis
252 was performed using the SuperScript® Double-Stranded cDNA Synthesis Kit (Invitrogen)
253 as per the manufacturer's instructions (NimbleGen Arrays User's Guide, Version 5.1) and
254 quantified with NanoDrop. Total cDNA was labeled overnight with the One-Color DNA
255 labeling kit (NimbleGen) as per the manufacturer's instructions. Arrays were scanned using
256 an Axon 4000B array scanner.

257 A *B. halodurans* C-125 Nimblegen custom array, containing a probe set of 22 unique 45mer-
258 60mer oligonucleotide probes for each of the 4066 genes of this bacterium, was used.
259 NimbleScan software (v 2.6.0.0, Roche NimbleGen) was used to generate one normalized

value per probe set using the RMA algorithm (background correction, normalization and summarization; data not logged). The data were then imported into R (34) using the limma package (35) and log2-transformed. Statistical analysis for differential expression between the mutant and wild-type groups was performed, taking into account the correlation due to processing batch (36, 37). Raw p-values were corrected for multiple hypotheses testing using the False Discovery Rate method (38). Annotation for the probe sets was primarily provided by Nimblegen and included BH ids (e.g., BH0001), gene names, descriptions, genomic locations and URL links to NCBI. Entrez Gene IDs and official gene symbols were downloaded from the *B. halodurans* genome record in NCBI (NC_002570). For analysis, we filtered to identify those genes that were altered by at least a 1.5-fold change in transcription (up-regulation and down-regulation). For data mining, we used DAVID bioinformatics resources that consist of an integrated biological knowledge base and analytic tools that use the results from the statistical analysis to explore and interpret gene regulation data (39).

274

275 **Results**

276 **Sublancin displays sub micromolar MICs against *Bacillus* strains**

277 The antibacterial activity of sublancin against selected *Bacillus* strains was first determined
278 by solid agar diffusion assays containing sublancin at a range of concentrations (0.097 μM –
279 50 μM). After confirmation that *B. subtilis* ATCC 6633 and *B. halodurans* C-125 were
280 sensitive, the minimum inhibitory concentrations (MICs) were determined by the broth
281 dilution method (40, 41). A series of dilutions of sublancin (0.097 μM – 50 μM) were made
282 and incubated with a defined number of bacterial cells in LB medium. Plates were
283 incubated for 18-24 h at 37 °C, and growth was assessed by measuring the optical density
284 of each well at O.D._{600nm}. The MICs were determined by fitting the data to a dose-response
285 curve. The MICs of sublancin against *B. halodurans* C-125 and *B. subtilis* ATCC 6633, in
286 liquid cultures, were 0.312 μM and 0.625 μM , respectively (Fig. S1 in the supplemental
287 material).

288

289 **Bactericidal activity of sublancin**

290 One element for consideration, when trying to understand how an antimicrobial compound
291 functions, is whether it is bactericidal or bacteriostatic. Furthermore, some bacteria lyse
292 after being killed, others lyse immediately, and yet others undergo non-lytic death (42, 43).

293 The ability of sublancin to kill or arrest the sensitive *Bacillus* strains was therefore
294 evaluated. *B. subtilis* ATCC 6633 and *B. halodurans* C-125 cultures were grown to mid log
295 phase, transferred to a 48-well plate and exposed to sublancin (1x and 4x MIC). After the
296 addition of sublancin, the O.D._{600nm} was monitored periodically. After a 6-hour incubation
297 period, the *B. halodurans* C-125 and *B. subtilis* ATCC 6633 cultures showed a decrease in
298 optical density, suggesting sublancin has bactericidal activity (Fig. 1). To verify whether
299 sublancin's activity was bactericidal, CFUs were determined by plating, which confirmed
300 the bactericidal activity observed by O.D. readings. The decrease in optical density was not
301 nearly as large as the decrease in CFUs, which implies that sublancin kills without
302 immediate lysis.

303

304 **Sublancin does not affect the integrity of the cell membrane**

305 Some bacteriocins destabilize the membrane or form pores (4, 44). Nisin is the prototypical
306 pore-forming bacteriocin, which binds to lipid II within the membrane (2). To determine
307 whether sublancin affects membrane integrity, we challenged cultures of *B. subtilis* ΔSPβ, *B.*
308 *subtilis* ATCC 6633 and *B. halodurans* C-125 with several different concentrations of
309 sublancin. We monitored the membrane integrity of *B. subtilis* ATCC 6633 and *B.*
310 *halodurans* C-125 by flow cytometric analysis, using the cell impermeable propidium iodide
311 (PI) dye, after a 30 min exposure to sublancin. Our nisin control showed an increase in
312 fluorescence due to membrane permeabilization, but sublancin did not, even at
313 concentrations as high as 32xMIC for *B. subtilis* ATCC 6633 and 64xMIC for *B. halodurans* C-
314 125 (Fig. 2). We monitored the membrane integrity of *B. subtilis* ΔSPβ with the
315 LIVE/DEAD® BacLight™ bacterial cell viability assay at 30 and 90 min after addition of
316 sublancin (Fig. S2 in the supplemental material). At both time points we found no change in
317 membrane integrity. When the same strain was exposed to nisin as a positive control, a
318 dramatic loss of membrane integrity was seen already after 30 min incubation. Collectively,
319 these experiments show that sublancin does not affect membrane integrity and likely acts
320 through an alternative mechanism.

321

322 **Resistance frequency**

323 The manifestation of antibiotic resistance to clinically used antibiotics suggests that
324 resistance is likely to develop against any antibacterial compound. It is useful however to
325 analyze the frequency at which resistance to novel antibacterial compounds arises (45).

326 The spontaneous resistance frequency is defined as the number of resistant mutants per
327 total number of viable cells that grow after an established period of time. The resistance
328 frequency of sublancin was determined by plating aliquots of bacterial culture onto agar
329 containing the antibacterial compound at 4xMIC. Aliquots were also plated onto agar plates
330 with no antibiotic to determine the number of viable bacterial cells in the liquid culture.
331 The resistance frequencies determined were relatively high, with resistance frequencies of
332 10^{-5} for *B. halodurans* C-125 and 10^{-6} for *B. subtilis* ATCC 6633. To verify that colonies
333 observed were indeed resistant to the antibiotic, they were sub-cultured in sublancin-free
334 LB media and plated on LB agar containing the antibacterial compound at a concentration
335 of 4xMIC. For both strains, the plated resistant strains grew a full lawn.

336

337

338 Identification of *B. subtilis* chromosomal regions that affect sensitivity to sublancin

339 We aimed at finding genetic factors that affect sensitivity to sublancin. To do this we first
340 employed the set of deletion mutants described by Tanaka et al. (29). These mutants were
341 created in a strain in which the prophages of *B. subtilis* had been deleted, including SP β .
342 Therefore, all mutant strains lack the gene encoding the immunity protein for sublancin,
343 *sunI* (46), making it an ideal collection of mutants for identifying interesting genomic
344 regions with respect to sublancin sensitivity. During the screening, we used LB agar
345 without NaCl, as it was previously shown that *B. subtilis* is more sensitive to sublancin in
346 low osmotic conditions (23). The strains were plated in duplicate on the LB agar and
347 spotted with 2 μ L of an overnight culture of the sublancin-producing strain *B. subtilis* 168.
348 We found strain JJS-DIn010, in which *rsiW* and *sigW* are deleted, to have increased
349 sensitivity (i.e. a larger zone of clearing around the producing colony) (Fig. 3a). This finding
350 is in concordance with previously reported observations (25), suggesting that our assay
351 was able to identify strains with altered sensitivity. Another strain was identified (JJS-
352 DIn042), in which the genes *ykvS*, *ykvT*, *ykvU*, *stoA*, *zosA*, *ykvY*, *ykvZ*, *glcT*, *ptsG*, *ptsH* and *ptsI*
353 were deleted. JJS-DIn042 was resistant to the effects of sublancin (Fig. 3a) under conditions
354 where the Δ SP β strain did not survive. Because of this interesting observation, we
355 investigated this region further by constructing several different individual gene deletion
356 mutants. This approach revealed that only the deletion of the *pts* operon (*ptsGHI*) results in
357 resistance to sublancin (Fig. 3b and Table 2). In contrast, a deletion of *glcT*, which plays a
358 regulatory role in the *pts* operon (47), did not result in sublancin resistance (Fig. 3b). PtsG
359 is the major glucose transporter of the phosphotransferase system (48), and PtsH and PtsI
360 are general components of the sugar transport system that phosphorylates the incoming
361 sugar (49). PtsH is more commonly known as HPr, and we will refer to it as such in this
362 work; PtsI is also called EI. In *B. subtilis*, the PTS transfers a phosphate group from
363 phosphorylated PtsI to HPr, which in turn transfers the phosphate to a variety of different
364 PTS permeases. For utilization of glucose, HPr transfers the phosphate to the IIA domain of
365 the sugar permease PtsG. The IIA domain then phosphorylates the IIB domain of PtsG,
366 which in turn transfers the phosphate to the incoming sugar. Lastly, the phosphorylated
367 sugar moves into glycolysis. It is intriguing that the PTS was identified in our screen for
368 sublancin sensitivity, as the most common PTS substrate is glucose whereas sublancin is
369 glucosylated. A functional homologue of HPr is present in *B. subtilis*, i. e. Crh. We therefore
370 tested a *crh* deletion mutant in the presence of sublancin, but no change in sensitivity was

371 observed compared to the wild-type (data not shown), suggesting the sensitivity to
372 sublancin is specifically dependent on HPr.

373

374 **Comparative genomics**

375 Bacteria often acquire stable resistance to antibiotics due to gene mutations. A comparative
376 genomics analysis was therefore performed to identify the mutations that conferred
377 resistance to *B. halodurans* C-125 after exposure to sublancin. The gDNA of the sensitive *B.*
378 *halodurans* C-125 and of four of the spontaneous resistant mutants obtained as described
379 above was extracted and sequenced using a HiSeq2000 Illumina sequencer. The wild-type
380 *B. halodurans* strain was mapped to the published *B. halodurans* C-125 genome sequence
381 (accession no. NC_002570.2) to generate a consensus sequence that was used for SNP
382 detection in sublancin-resistant mutants of *B. halodurans*. Comparison of gDNA of the wild-
383 type sensitive strain with the four sublancin resistant mutants revealed several mutations.
384 One strain contained three mutations in the intergenic region between the transcriptional
385 anti-terminator (Locus tag: BH0845) and *ptsG* (Locus tag: BH0844), another strain
386 contained a missense mutation in the gene for mannitol-1-phosphate 5-dehydrogenase
387 (Locus tag: BH3851), and most interestingly, the three strains that did not have a mutation
388 in the intergenic region mentioned above all had non-sense mutations in the gene for the
389 glucose-specific transporter subunit IIC that is part of the multidomain protein PtsG (Table
390 S2 in the supplemental material). The missense mutation prevents production of PtsG, and
391 the three mutations in the intergenic region between the antiterminator and *ptsG* are
392 predicted to considerably stabilize the structure of the terminator (Fig. S3 in the
393 supplemental material), thus potentially also preventing *ptsG* transcription. Once more,
394 these findings point to the PTS being important for sensitivity to sublancin.

395

396 **Gene expression profile by microarray analysis of *B. halodurans* C-125**

397 Antimicrobial resistance mutants provide valuable insights, but the information obtained
398 from a resistance phenotype is not always representative of the identity of the target. We
399 therefore also monitored changes in global gene expression upon exposure of *B. halodurans*
400 C-125 to sublancin.

401 The expression profiles revealed four genes that are part of sulfur metabolism that are
402 highly up-regulated (changes from 9-14 fold, Table S3 in the supplemental material). The
403 analysis also revealed up-regulated genes involved in transmembrane transporter
404 activities, whereas genes involved in amino sugar and nucleotide sugar metabolism were
405 up- and down-regulated. Interestingly, the genes for HPr and for PtsG that were also
406 identified in the set of deletion mutants and in the comparative genomics analysis were
407 down-regulated (Table S3 in the supplemental material) as was another PTS protein that is
408 homologous to YpqE in *B. subtilis*, a putative phosphotransferase enzyme IIA component
409 (50). These data again suggest that, like in *B. subtilis*, the PTS is involved in the sensitivity of
410 *B. halodurans* towards sublancin.

411

412 **Addition of PTS sugars to the growth media results in increased resistance to**
413 **sublancin**

414 Several bacteriocins have previously been shown to target PTS proteins as part of their
415 mode of action. In these reported cases, addition of the relevant sugar resulted in an
416 increased sensitivity to the bacteriocin (6, 7, 11). This effect is due to elevated uptake of the
417 respective bacteriocins via the PTS. We therefore investigated whether this was also true
418 for sublancin. The PTS is able to use many sugars, employing a different transporter for
419 each sugar together with the HPr and PtsI proteins. Once the sugar is phosphorylated, it
420 moves into glycolysis at the relevant metabolic junction. To investigate the influence of
421 added sugars, *B. subtilis* Δ SP β cultures were diluted in LB media (without NaCl) containing
422 different sugars and grown in 96-well microtiter plates with shaking to an O.D._{600nm} 0.5
423 before addition of sublancin at the MIC of 200 nM, as measured for this strain under these
424 conditions. The presence of the PTS sugars glucose, sucrose and fructose, prevented the
425 growth inhibition imposed by sublancin (Fig. 4a) since no significant reduction in O.D. was
426 observed. In contrast, the non-PTS sugars citrate, galactose and succinate had no influence
427 on sublancin's activity (Fig. 4b). The two exceptions were the non-PTS sugars glycerol and
428 malate. In this respect it is noteworthy that the the glycerol kinase GlpK requires

phosphorylation by HPr for glycerol utilization (48, 51). Malate is a preferred carbon source for *B. subtilis* and is known to influence the carbon catabolite repression response (52). In this context it is noteworthy that a decrease in antimicrobial activity was also reported for glycocin F upon supplementation of the media with GlcNAc, which is the sugar that is attached to glycocin F at two positions (17).

To further delineate the effects of sugars on sublancin sensitivity, purified sublancin was spotted onto lawns of *B. subtilis* Δ SP β , which were grown on agar plates containing the defined M9 minimal medium supplemented with glucose, malate or citrate. When glucose was present the cells were always resistant to sublancin. In contrast, with citrate a large zone of clearing was observed. In the presence of malate an intermediately sized zone of inhibition was observed. This observation underpins the view that the carbon source affects the sensitivity to sublancin (Fig. S4 in the supplemental material).

Since glucose was found to prevent the effect of sublancin, we wondered whether it would be possible to rescue sublancin-treated cells by addition of glucose. We therefore grew the bacteria on LB medium (with NaCl) and added sublancin at O.D._{600nm} 0.5. The cells were then incubated for 30 min before addition of the same PTS and non-PTS sugars as in the previous experiment (Fig. 4c and d). Glucose almost instantaneously rescued the cells from the growth inhibition that sublancin imposed on the cells. Fructose also rescued the cells, but to a smaller extent than glucose. The non-PTS sugar glycerol rescued the cells in a similar manner to fructose. Malate was also able to rescue the cells, but this took approximately 100 min following the addition of the sugar, whereas the effect for glycerol and fructose was observed immediately after the addition of the respective sugar. In contrast, the addition of the other non-PTS sugars or sucrose had no effect on the survival of the bacteria.

The observed rescue of growth by addition of the different PTS sugars and glycerol suggests that the PTS transporter is not being irreversibly inactivated by sublancin, but perhaps instead sublancin affects the pathway that leads to phosphorylation of the sugar. The addition of sublancin and the PTS sugar at the same time could result in competition for phosphorylation of the sugar or the glucose on sublancin (or its metabolite). With this in mind we looked at the phosphorylation sites on the HPr protein.

Phosphorylation of HPr is responsible for sensitivity to sublancin

461 The HPr protein is phosphorylated on two sites. The first, His15, is phosphorylated by PtsI.
462 HPr then transfers the phosphate group to PtsG, and the phosphate is subsequently used to
463 phosphorylate the incoming sugar. The second, Ser46, is phosphorylated by HPr kinase
464 (HPrK) in conditions of large glycolytic flux. This phosphorylation subsequently allows HPr
465 to form a complex with the catabolite control protein A (CcpA). This HPr-CcpA complex
466 mediates carbon catabolite repression by binding to its cognate operator regions. To link
467 sublancin sensitivity to one of these HPr-mediated processes, we tested two *B. subtilis* Δ SP β
468 strains with point mutations at one of the two HPr phosphorylation sites. As shown in Fig.
469 5a, *B. subtilis* Δ SP β carrying the S46A point mutation in HPr was fully sensitive to sublancin.
470 In contrast, the strain carrying the H15A point mutation in HPr displayed an increased level
471 of resistance to sublancin. This observation suggests that *hprK* and *ccpA* deletion mutants
472 would remain sensitive to sublancin, since the carbon catabolite-repressing function of HPr
473 is not affected. This prediction was indeed confirmed, as the deletion of either of these two
474 genes had no effect on sublancin sensitivity (Fig. 5b). Also, the addition of glucose to the
475 Δ *ccpA* mutant conferred resistance to sublancin (not shown), providing further evidence
476 that it is not the carbon catabolite-repressing branch of HPr regulation that leads to
477 sublancin sensitivity. Instead, it seems to be the PTS branch involving the H15
478 phosphorylation site that is responsible for the effects on sublancin sensitivity. However,
479 how phosphorylation of His15 of HPr is exactly tied to sublancin sensitivity is presently
480 unknown.

481

482 **Discussion**

483 Sublancin is a bacteriocin that was recently found to be glucosylated as part of its
484 maturation process and this glucosylation is required for activity (15). We show in this
485 study that sublancin is bactericidal and that it does not kill by pore formation or disruption
486 of membrane integrity. Four different lines of evidence point towards the
487 phosphoenolpyruvate:sugar phosphotransferase system as a factor affecting the activity of
488 the bacteriocin. Experiments with deletion mutants of *B. subtilis* identified PtsG, HPr, and
489 PtsI, but not GlcT and Crh, as important for sensitivity to sublancin. In addition, three of
490 four *B. halodurans* sublancin-resistant mutants contained stop-codon mutations within the
491 *ptsG* gene, with the fourth resistant strain having three mutations that potentially interfere
492 with *ptsG* expression. The transcriptional profile also indicated a strong down-regulation of

493 PTS genes upon exposure to sublancin and, lastly, addition of PTS sugars decreased the
494 sensitivity to sublancin.

495

496 The PTS has been previously described as affecting sensitivity to other bacteriocins,
497 including dysgalacticin and lactococcin A (6, 7, 53). Dysgalactin appears to bind to the
498 glucose and mannose transporters of the PTS (7). Dysgalacticin was shown to block the
499 uptake of glucose and the non-metabolisable analog 2-deoxyglucose, and also to perturb
500 the membrane of the cell causing the dissipation of membrane potential (7). This activity
501 appears to be different from the mechanism used by sublancin as addition of glucose or any
502 other PTS sugar blocked the killing activity of sublancin and membrane disruption was not
503 observed. Lactococcin A also uses components of the mannose PTS in its mode of action.
504 Lactococcin A binds to the membrane-located complex of the IIC and IID subunits of the
505 mannose transporter (6), resulting in dissipation of the membrane potential (44). Like the
506 observations with dysgalactin, decreased growth rates were observed for cells grown with
507 mannose or glucose as the sole carbon source in the presence of lactococcin A (6). The
508 current study suggests that sublancin is also functioning in a different manner to
509 lactococcin A, since the studies with gene deletion strains and spontaneous resistance
510 mutants both point at the phosphorylation components of the PTS as being key to
511 sublancin sensitivity rather than the membrane components. When we monitored
512 sublancin susceptibility using M9 minimal medium supplemented with glucose as the
513 single carbon source, cells were completely immune to the effects of sublancin. A third
514 bacteriocin, the circular molecule garvicin ML requires the maltose-binding protein for
515 activity. Growth in media where the sole carbon source is either maltose or galactose again
516 resulted in increased sensitivity to this bacteriocin (54).

517

518 Two regions of the *B. subtilis* chromosome have now been identified that result in
519 resistance to sublancin. The first being *mscL*, encoding a mechanosensitive channel as
520 described by Kouwen et al. (23), and in this work the proteins encoded by the *ptsGHI*
521 operon. Several scenarios may describe the mechanism by which sublancin is interacting
522 with the PTS. Firstly, it is intriguing that it is the glucose transporter that was identified,
523 given that sublancin is modified with an S-linked glucose moiety. The glucose moiety on
524 sublancin could potentially be recognised by the transporter to facilitate entry into the cell
525 or potentially to kill it through its interaction with this system. A competition between the

526 sublancin and glucose could explain the observed decrease in sensitivity upon addition of
527 glucose. We note that HPr and domains IIAB of PtsG are located in the cytoplasm (Table S4
528 in the supplemental material), and hence for this mechanism to be correct, the glucose on
529 sublancin would have to traverse the transporter or bypass the glucose transporter via the
530 MscL channel. In this respect it is noteworthy that the other sugars that were able to
531 protect the bacteria from sublancin are either gluconeogenic, or feed into glycolysis lower
532 down the pathway, therefore possibly bypassing the need for the glucose transporter.
533 When we tested strains that express variants of the HPr protein with point mutations that
534 remove the phosphorylation sites, the mutation that led to increased resistance to
535 sublancin was H15A. Phosphorylation of His15 is responsible for transferring a phosphate
536 group to the incoming PTS sugar. This points towards a critical role of phosphorylation in
537 the growth inhibition by sublancin and seems to suggest that sublancin may need to be
538 phosphorylated upon its entry into the cell in order to exert its bactericidal effect.
539 Interestingly, bacterial growth was rescued when PTS sugars were added to the growth
540 medium 30 min after the challenge with sublancin. This finding implies that either the
541 specific growth-inhibiting mechanism employed by sublancin is reversible, or that the
542 addition of the PTS sugars results in the cell using a different biological process that allows
543 survival.

544

545 In conclusion, we show that sublancin exerts its bactericidal effects in a novel manner. At
546 present it is not clear how exactly sublancin is interacting with the PTS and several
547 questions remain for future research. Is there a physical interaction between sublancin and
548 the PTS in the inhibited cells? Is there a link between the PTS and the MscL channel? How is
549 sublancin actually inhibiting growth of the cell? How does the strong structural similarity
550 of glycocin F and sublancin fit into the mechanism and what role does the three-
551 dimensional structure of the peptide components of these glycocins play (21, 22). In a time
552 where bacteria are becoming resistant to the antimicrobial compounds that we currently
553 use in clinical practice, research to understand how infections can be fought in alternative
554 manners is essential. The apparently novel mechanism by which sublancin kills sensitive
555 species of bacteria may therefore offer biological insights for the development of new
556 antimicrobial strategies. Whether the mechanism identified in *Bacillus* is also operational
557 in sensitive pathogens, such as *S. aureus* and *Listeria monocytogenes*, requires further in-
558 depth analyses.

559

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572

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789 **Figure Legends**

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791 **Figure 1. Growth inhibition of *B. subtilis* ATCC 6633 and *B. halodurans* C-125 by**
792 **sublancin 168.** (a) Time-dependent changes in O.D._{600nm} of cultures of *B. subtilis* ATCC
793 6633 in the absence (circles) or presence of sublancin 168 at 1xMIC (squares) and 4xMIC
794 (triangles). (b) Aliquots of the cultures in panel (a) were analysed for colony forming units
795 (CFUs). (c) Time-dependent changes in O.D._{600nm} of cultures of *B. halodurans* C-125. The
796 same symbols are used as in panel (a). (d) Aliquots of the cultures in panel (c) were
797 analysed for CFUs. The mean values of the data from one experiment conducted in
798 triplicate are shown. The data are representative of three independent experiments. Error
799 bars indicate standard deviations. When error bars are not visible, they were a smaller
800 than the size of the symbol used.

801 **Figure 2. Membrane integrity assays by measuring propidium iodide (PI) uptake.**

802 Addition of sublancin at the indicated concentrations (black bars) does not alter the
803 membrane permeability of (a) *B. halodurans* C-125 and (b) *B. subtilis* ATCC 6633. Nisin was
804 used as positive control (grey bars). * indicates a $P < 0.05$ between nisin (0.1 μM – 20 μM)
805 treated cells relative to no drug. In all experiments in which the cells were exposed to
806 sublancin, the increase in MFI relative to control was not statistically significant ($P > 0.05$).
807 The means of the data from one experiment conducted in triplicate are shown. The data are
808 representative of those from three independent experiments. Error bars indicate standard
809 deviations.

810 **Figure 3. Deletion of the *ptsGHI* operon results in resistance to sublancin.** (a) The *B.*
811 *subtilis* strains described by Tanaka et al (29) were screened for increased and reduced
812 sensitivity to sublancin. The parental strain of the collection is labelled as 'master strain'. In
813 JJS-DIn042 the region from *ykvS* to *ptsI* is deleted and in JJS-DIn010 *rsiW* and *sigW* are

814 deleted. (b) Growth curves of mutant strains with individual deletions of the genes that are
815 responsible for the resistance in strain JJS-DIn042. Upper panel - Blue line - *B. subtilis*
816 Δ SP β , green line - *B. subtilis* Δ SP β -*glcT*. Lower panel - black line - *B. subtilis* Δ SP β -*ptsG*, red
817 line - *B. subtilis* Δ SP β -*ptsH*, orange line *B. subtilis* Δ SP β -*ptsI*. Solid lines - no sublancin.
818 Dotted lines - 200 nM sublancin added at 100 min as indicated by the vertical grey line.
819 Deletion of any of the genes within the *ptsGHI* operon results in resistance to sublancin.
820 Deletion of the gene encoding the transcriptional anti-terminator *glcT* has no effect on the
821 sensitivity of *B. subtilis* to sublancin. The means of the data from one experiment conducted
822 in triplicate are shown. The data are representative of those from three independent
823 experiments.

824 **Figure 4. Addition of PTS sugars to LB blocks growth inhibition by sublancin (a)**

825 Growth curves of *B. subtilis* Δ SP β in LB medium with salt with added PTS sugars. Blue line -
826 no addition of sublancin, red line - addition of sublancin, black solid line - addition of 0.3%
827 glucose, black dotted line - addition of 0.3% fructose, black long dashed line - addition of
828 0.3% sucrose, black short dashed line - addition of 0.3% glycerol. Sublancin was added at
829 120 min as indicated by the vertical grey line. (b) Growth curve of the Δ SP β strain in LB
830 medium with addition of non-PTS sugars. Blue line - no addition of sublancin, red line -
831 addition of sublancin, green line - addition of 0.4% malate, grey short dashed line -
832 addition of 0.4 % citrate, grey dotted line - addition of 0.4% galactose, grey solid line -
833 addition of 0.4% succinate. (c) Growth curve of the Δ SP β strain with sublancin added at
834 150 min followed by the addition of PTS and non-PTS sugars 30 min later as depicted by
835 the two vertical lines, respectively. Blue line - no sublancin, blue dashed line - addition of
836 sublancin, black line - 0.3% glucose, purple line - 0.3% fructose, orange line - 0.3%
837 glycerol, green line - 0.4% malate, (final concentration of sugars shown). (d) Growth curve
838 of the Δ SP β strain with sublancin added at 150 min followed by the addition of PTS and

839 non-PTS sugars 30 min later as depicted by the two vertical lines, respectively. Blue line –
840 no sublancin, blue dashed line – addition of sublancin, grey line – 0.4% citrate, red line –
841 0.3% sucrose, pink line – 0.4% galactose, green line – 0.4% succinate (final concentration of
842 sugars shown). The means of the data from one experiment conducted in triplicate are
843 shown. The data are representative of those from three independent experiments.

844 **Figure 5. The H15A mutation in HPr results in increased resistance to sublancin.** (a)

845 The two phosphorylation sites in the HPr protein were mutated individually to alanine
846 residues. The growth curves of the resulting strains are shown, with 200 nM sublancin
847 added at 120 min as depicted by the vertical line. Blue line - *B. subtilis* Δ SP β , grey line - *B.*
848 *subtilis* Δ SP β -H15A, black line - *B. subtilis* Δ SP β -S46A. Solid line – no sublancin. Dashed line
849 – plus sublancin. (b) Blue line - *B. subtilis* Δ SP β , grey line - *B. subtilis* Δ SP β -*hprK*, black line -
850 *B. subtilis* Δ SP β -*ccpA*. Solid line – no sublancin. Dashed line – plus sublancin. The *AccpA* and
851 *ΔhprK* mutations have no effect on the sensitivity of the cells to sublancin. 200 nM
852 sublancin added at 120 min. The means of the data from one experiment conducted in
853 triplicate are shown. The data are representative of those from three independent
854 experiments.

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Table 1. Strains

Strain	Genotype	Reference
168	Wild-type <i>B. subtilis</i> strain	Laboratory Collection
Δ SP β	Wild-type <i>B. subtilis</i> 168 strain lacking the entire SP β prophage	(14)
C-125	Wild-type <i>B. halodurans</i>	(55)
ATCC 6633	Wild-type <i>B. subtilis</i>	(56)
168 Deletion collection	Collection of <i>B. subtilis</i> mutants lacking large genomic regions	(29)
Δ SP β ::cat	Chloramphenicol selectable Δ SP β prophage mutant of <i>B. subtilis</i>	(14)
Δ yvkS-yvkW	<i>B. subtilis</i> Δ yvkS-yvkW::phleo	This study
Δ ykvY-glcT	<i>B. subtilis</i> Δ ykvY-glcT::phleo	This study
Δ ptsG-ptsI	<i>B. subtilis</i> Δ ptsG-ptsI::phleo	This study
Δ SP β -QB5435	<i>B. subtilis</i> Δ pstG::cat; QB5435 \rightarrow SP β	(47)
Δ SP β -MZ303	<i>B. subtilis</i> Δ ptsH::cat; MZ303 \rightarrow SP β	(57)
Δ SP β -GP864	<i>B. subtilis</i> Δ ptsI::ermC; GP864 \rightarrow SP β	(58)
Δ SP β -QB5407	<i>B. subtilis</i> Δ ccpA::spec; QB5407 \rightarrow SP β	(59)
Δ SP β -GP202	<i>B. subtilis</i> Δ hprK::spec; GP202 \rightarrow SP β	(60)
Δ SP β ::cm-GP506	<i>B. subtilis</i> ptsH H15A; SP β cm \rightarrow GP506	(61)
Δ SP β ::cm-GP507	<i>B. subtilis</i> ptsH S46A; SP β cm \rightarrow GP576	(62)

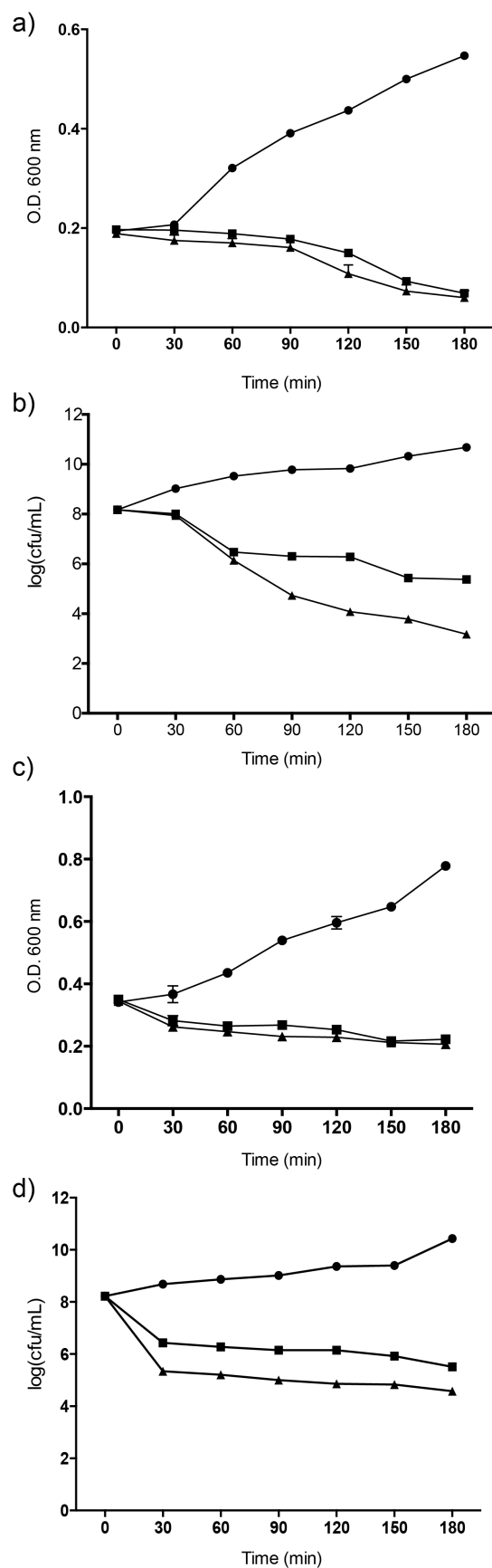
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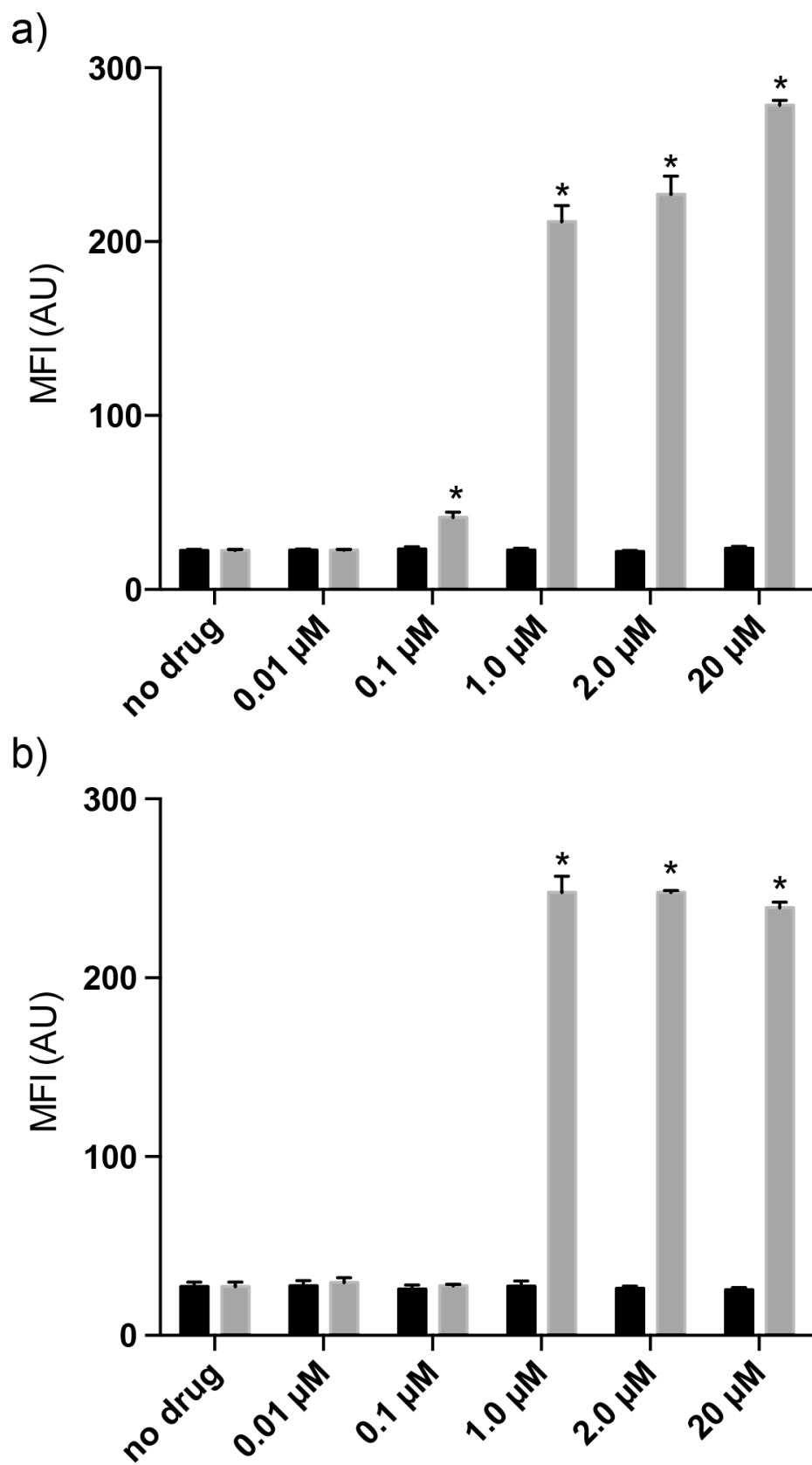
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864 **Table 2. Phenotype of single gene deletion strains of *B. subtilis* Δ SP β upon**
865 **exposure to sublancin.**

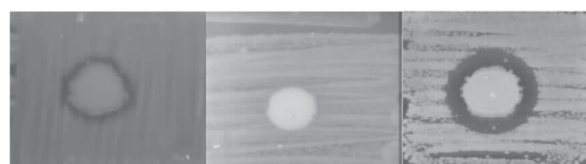
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Mutation	Sublancin resistant or sensitive
<i>ykvS</i>	Sensitive
<i>ykvT</i>	Sensitive
<i>ykvU</i>	Sensitive
<i>stoA</i>	Sensitive
<i>zosA</i>	Inconclusive
<i>ykvY</i>	Sensitive
<i>ykvZ</i>	Sensitive
<i>glcT</i>	Sensitive
<i>ptsG</i>	Resistant
<i>ptsH</i>	Resistant
<i>ptsI</i>	Resistant





a)

Master
strainJJS-
DIn042JJS-
DIn010

b)

